

## NOTES

# Proteolytic Processing of the Ebola Virus Glycoprotein Is Not Critical for Ebola Virus Replication in Nonhuman Primates<sup>∇</sup>

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**Enveloped viruses often require cleavage of a surface glycoprotein by a cellular endoprotease such as furin for infectivity and virulence. Previously, we showed that Ebola virus glycoprotein does not require the furin cleavage motif for virus replication in cell culture. Here, we show that there are no appreciable differences in disease progression, hematology, serum biochemistry, virus titers, or lethality in nonhuman primates infected with an Ebola virus lacking the furin recognition sequence compared to those infected with wild-type virus. We conclude that glycoprotein cleavage by subtilisin-like endoproteases is not critical for Ebola virus infectivity and virulence in nonhuman primates.**

*Ebola virus*, a member of the family *Filoviridae*, causes hemorrhagic fever in human and nonhuman primates, with up to 90% lethality (2, 9). Although the molecular basis for the extreme virulence of Ebola virus remains largely unknown, many enveloped viruses require cleavage of their surface glycoprotein by cellular endoproteases for infectivity and virulence (3). This cleavage event produces two subunits and exposes a fusion peptide that inserts into the host cell membrane, thereby “fusing” the cellular and viral membranes. The glycoprotein (GP) of *Zaire ebolavirus* (ZEBOV) contains a highly conserved consensus sequence for the subtilisin-like endoprotease furin (10, 12). ZEBOV GP cleavage by this protease has been previously demonstrated (12). By contrast, the glycoprotein of *Reston ebolavirus*, the species least pathogenic for humans, lacks an optimal furin cleavage site (containing lysine, but not arginine, at position –4). Furin cleavage was therefore considered an important determinant of Ebola virus virulence (2). However, studies of pseudotyped viruses expressing Ebola GP with an altered furin recognition sequence demonstrated that GP cleavage by furin is dispensable for in vitro infectivity of the pseudotyped viruses (7, 14).

Using reverse genetics, which allows the artificial generation of Ebola virus from cloned cDNA (8, 13), we previously generated a ZEBOV mutant based on the Mayinga strain in which the multibasic sequence motif for furin cleavage (RRTRR at amino acids 497 to 501 of GP) was replaced with nonbasic

amino acids (AGTAA) (8). This mutation prevented the generation of GP1 and GP2, the two cleavage products, and yet the mutant virus grew in cell culture to titers similar to those of wild-type virus, albeit with mildly attenuated growth early in infection (8). These findings indicated that furin-mediated cleavage of Ebola virus GP is not essential for virus replication in cell culture. However, does this finding also hold true in animals?

Nonhuman primates, including cynomolgus and rhesus macaques, develop disease with a pathology that resembles that observed in Ebola virus-infected humans. Because these animals display similar symptoms and disease progression and succumb to infection with wild-type ZEBOV without prior adaptation, they are considered the “gold standard” animal model for Ebola virus infection.

Here, we assessed our recombinant Ebola virus that lacks the conserved furin recognition sequence (ZEBOV $\Delta$ Cleav) in rhesus macaques. Two healthy, filovirus-seronegative, adult rhesus macaques were inoculated in the right caudal thigh with 10<sup>3</sup> PFU of recombinant ZEBOV $\Delta$ Cleav virus. Two additional animals were inoculated with the same dose of wild-type ZEBOV generated by reverse genetics. Animals were observed twice daily for signs of illness, including cutaneous rashes, hemorrhage, and reduced activity. Blood samples for virus titration, hematology, and serum biochemistry were collected prior to infection and on days 3, 4, 6, and 7 postinfection. Animals that showed severe signs of disease were euthanized in compliance with approved protocols. Animal studies were performed under biosafety level 4 biocontainment conditions at the U.S. Medical Research Institute of Infectious Diseases and approved by the U.S. Medical Research Institute of Infectious Diseases Laboratory Animal Care and Use Committee.

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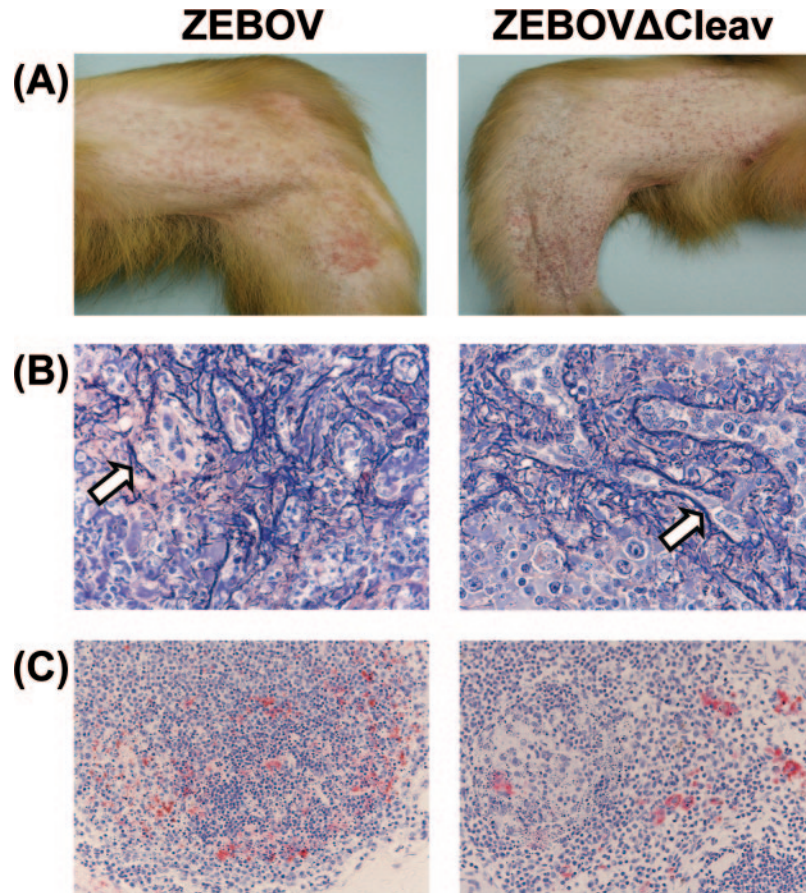


FIG. 1. Comparison of monkeys infected with ZEBOV (left panels) or ZEBOV $\Delta$ Clev (right panels). (A) Macular cutaneous rashes on day 6 postinfection. (B) Phosphotungstic acid hematoxylin-positive fibrin in spleen. Staining was carried out as described in reference 5. Note that there is no apparent difference in the amount or distribution of polymerized fibrin (see arrows that point to fibrin-stained regions). Original magnification,  $\times 40$ . (C) Immunostaining of inguinal lymph nodes. Staining was carried out as described in reference 4. Note that positive immunostaining of monocytes-macrophages for Ebola virus (red) is evident in both animals. Also, lymphoid depletion and lymphocytolysis are prominent in both animals. Original magnification,  $\times 20$ .

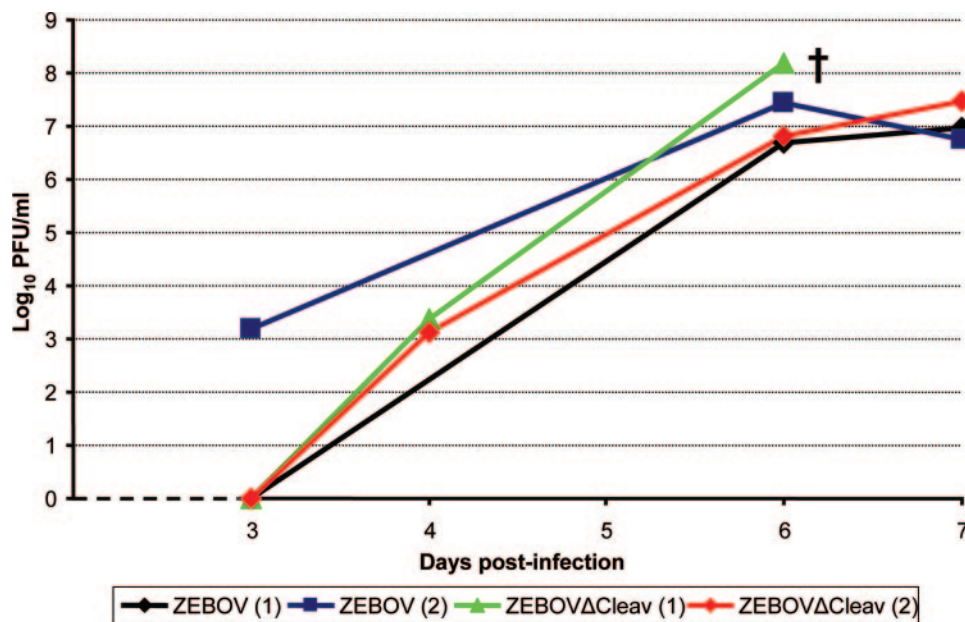


FIG. 2. Viremia in rhesus macaques infected with wild-type Ebola virus (ZEBOV) generated by reverse genetics and in a mutant lacking the conserved recognition sequence for furin (ZEBOV $\Delta$ Clev). Note that one animal infected with ZEBOV $\Delta$ Clev expired on day 7 postinfection ( $\dagger$ ); therefore, no fresh serum sample for that animal could be obtained for virus titration.

TABLE 1. Virus titers in various organs of rhesus macaques infected with wild-type Ebola virus (ZEBOV) or with an Ebola virus lacking the consensus furin recognition motif in GP (ZEBOV $\Delta$ Cleav)

Virus strain (animal no.)	Virus titer ( $\log_{10}$ of PFU/gram tissue on day 7 postinfection) <sup>a</sup>													
	Liver	Spleen	Kidney	Lungs	Pancreas	Brain	Heart	Bone marrow	Testis	Adrenal glands	Axillary lymph nodes	Inguinal lymph nodes	Mesenteric lymph nodes	Mandibular lymph nodes
ZEBOV (1)	6.93	7.41	5.51	5.50	2.40	3.98	4.93	6.15	5.81	6.01	6.20	7.12	6.68	6.01
ZEBOV (2)	6.95	7.40	6.70	6.10	6.51	5.01	5.31	6.08	6.75	7.07	6.79	6.47	7.86	6.90
ZEBOV $\Delta$ Cleav (1)	5.54	2.87	6.10	6.74	0	6.22	5.97	5.90	6.22	5.65	6.37	6.26	5.89	6.30
ZEBOV $\Delta$ Cleav (2)	7.80	7.70	5.87	4.23	5.65	5.31	5.83	6.90	6.60	7.33	6.76	6.59	7.44	7.05

<sup>a</sup> Animals were inoculated in the caudal thigh with  $10^3$  PFU of virus.

Animal research was conducted in compliance with the Federal statutes and regulations relating to animals and experiments involving animals, adhering to the principles in the *Guide for the Care and Use of Laboratory Animals* (7a). The facility used is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

All infected animals were asymptomatic until day 4 postinfection, when all four animals became febrile (temperature > 39.7°C). By day 5, all four had developed characteristic macular cutaneous rashes (Fig. 1A). On day 7, one animal infected with ZEBOV $\Delta$ Cleav expired, while the remaining three monkeys became terminally ill and were euthanized. These findings demonstrate that wild-type ZEBOV generated by reverse genetics is as virulent as the original ZEBOV (4) and that Ebola virus lacking the consensus furin recognition motif in GP is as capable as wild-type virus of causing fatal disease in nonhuman primates.

No appreciable differences in hematology and serum biochemistry (measured as described previously; see reference 4) were detected between monkeys infected with ZEBOV virus and those infected with ZEBOV $\Delta$ Cleav virus. In similarity to results previously obtained for Ebola virus infections in cynomolgus macaques (4, 6), all animals developed lymphopenia and thrombocytopenia with elevated liver enzyme levels during late infection. The most pronounced increases were in alanine aminotransferase and aspartate aminotransferase levels on days 6 and 7 postinfection, indicative of liver failure. In addition, monkeys infected with ZEBOV or ZEBOV $\Delta$ Cleav virus revealed similar levels of fibrin deposits (Fig. 1B) and similar numbers of infected monocytes-macrophages (Fig. 1C).

The clinical signs and blood biochemistry of rhesus macaques infected with ZEBOV $\Delta$ Cleav virus suggested replication of the virus in monkeys; this growth may, however, have been attenuated due to the lack of the consensus furin recognition motif in GP. We, therefore, determined virus titers in serum and organs. When plaque assays were performed, virus titers in serum were undetectable or low on day 3 postinfection but reached high levels on days 6 and 7 postinfection (6.7 to 7.5  $\log_{10}$  PFU/ml) (Fig. 2) for ZEBOV-infected animals. The two animals infected with ZEBOV $\Delta$ Cleav reached similar titers (6.8 to 8.2  $\log_{10}$  PFU/ml) on day 6 and 7 postinfection, respectively. Note that one animal infected with ZEBOV $\Delta$ Cleav expired on day 7 prior to blood sampling. We also conducted quantitative real-time reverse transcription-PCR and found low numbers of genomic copies on day 3 postinfection for animals infected with ZE-

BOV and no viral genomic copies in animals infected with ZEBOV $\Delta$ Cleav (note that the detection limit of the assay is 140 genomic copies), suggesting mild attenuation of the mutant virus. Quantitative reverse transcription-PCR analysis on day 6 postinfection revealed no differences in the numbers of genomic copies between the two groups.

ZEBOV and ZEBOV $\Delta$ Cleav also replicated to comparable titers in various organs (Table 1), although viral titers were low in the spleen and undetectable in the pancreas of the animal infected with ZEBOV $\Delta$ Cleav that died on day 7. Virus titers in the pancreas and brain of one ZEBOV-infected monkey were also low, suggesting normal variability among these infected animals. Our findings establish that Ebola virus replication in nonhuman primates is not significantly attenuated by the loss of the furin recognition motif in GP.

To confirm that the amino acid changes introduced into the furin recognition motif of ZEBOV $\Delta$ Cleav were retained during replication, we sequenced the GP genes of viruses from plasma and spleen samples of ZEBOV $\Delta$ Cleav-infected animals on day 6 and day 7 postinfection, respectively. For all samples, the introduced mutations were retained. Moreover, no other nucleotide replacements were found in the GP gene.

GP cleavage by host cell proteases is critical for the virulence of many viruses, and the conservation of the furin cleavage motif among Ebola virus GP sequences suggested a similar role in Ebola virus replication. Our previous and present findings, however, establish that the furin recognition sequence in Ebola virus GP is dispensable in cell culture (8) and in nonhuman primates (this study), indicating that GP cleavage is dispensable for Ebola virus replication in these systems. However, GP cleavage by furin or a furin-like endoprotease may be required for Ebola virus replication in its natural host. GP1 degradation by endosomal cathepsins is important for the initiation of Ebola virus uncoating (1, 11). Our findings indicate that GP cleavage by furin into GP1 and GP2 is not a prerequisite for cathepsin digestion. Whether proteolytic processing by other proteases in endosomes is required prior to cathepsin digestion remains unknown.

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